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Infection monitoring in wounds

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Abstract

Wound infection monitoring is a challenging task. It is only solvable by designing an integrable and cost-efficient sensor which measures a relevant set of parameters. One viable parameter is the formation of neutrophil extracellular traps (NETs). Their task is trapping pathogens in the wound. A wound infection results in massive release of them which can be detected with impedimetric methods. Our investigations focused on the characterization of the biological process with an *in vitro* model. The model environment is a cell culture with neutrophil granulocytes cultured on interdigitated electrodes which represent the sensor surface. Detected impedance changes caused by NET-formation were in the range of 35 % and even higher. This implies that impedance measurements are suitable for NET detection. We derived a measurement and evaluated it by differing conditions like changing stimulation agent and varying the cell number. For both conditions the results of impedance and phase angle deviation can be confirmed. In combination with other parameters a sensor can be designed for specific detection of wound infections. These aspects are integrated in our sensor concept.

Keywords: impedimetric sensor; wound monitoring, neutrophil extracellular traps; impedance spectroscopy

1. Introduction

Intensive wound care is an important issue for the treatment of poor-healing chronic wounds. Such wounds have a high prevalence with about 18 million patients worldwide [1]. One of the major tasks in wound treatment is monitoring the infection status of the wound. Treatment standards differ regionally.

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However, modern treatments commonly use hydroactive wound covers. Their great benefits are a longer retention period, a good healing rate and less pain during cover change. Monitoring the infection status means detaching the wound cover, visual inspection and checking the wound exudate on germ contamination by a smear test. All this procedure involves a lot of pain and intensive stress for the wound milieu. The healing itself is disturbed strongly. To tap the full potential of the hydroactive treatment an *in vivo* infection monitoring is required.

This measurement task is complex due to the great variety of the biological process of infection with a set of multiple parameters. Clinical practice includes some empirically determined parameters like temperature and pH. Also subjective criteria like wound size, color and odor are relevant. Some experimentally ascertained quantities are germ quantity and constituent parts of leukocytes determined by microscopy methods, hydrogen peroxide, lactate and the amount of exudate. Some of these wound properties are summarized in table 1 with relevant measurement ranges.

Table 1. Parameters for wound monitoring

Parameter	Normal	Pathological
pH [2]	Not clarified yet	Permanently higher then 7.5
Temperature [3, 4]	Depending on wound location and type	Increase of 2.8 … 3.6 K
Neutrophil extracellular traps (NETs) [5, 6]	None	Massive release, not quantified yet
Hydrogen peroxide [7, 8]	Below 5 µM	5 … 15 µM *in vitro*
Lactate [9, 10]	At the beginning: 4 … 12 mM, from the 7th day up to 15 mM	Just an indicator for the healing status
Amount of exudate [11]	Low exuding	Approximately 862 g/m²

One approach for a wound sensor by Vincenzini *et al.* [12] is based on a fiberoptical set-up with functional coatings on these fibers. The addressed parameters are pH and C-reactive protein (CRP). The functional layer selectively changes its optical properties, especially its refractive index which can be detected by a spectrometer. The materials used as functional coatings are on the one hand pH-responsive hydrogels and on the other hand dextran polymers with a covalently bound receptor against CRP. The optical set-up requires high demands on precise manufacturing of the sensor and seems extensive for the application in wounds. The need for a spectrometer excludes a wireless solution. The bulky sensor head does not fit demands concerning planarity especially for pain sensitive wounds. Another concept is described by McColl and Connolly *et al.* [11, 13]. This group developed an impedimetric sensor to measure wound moisture with planar printed electrodes. Wound moisture is an important parameter, but its specificity in infection detection is unsatisfactory. Nevertheless, this set-up is one of the promising approaches for wound status determination because of its easy utilization method and good integratability. Impedance detection is a very sensitive method which is strongly related to the electrode configuration. Concerns can be found because of the insufficient miniaturization. The large-scale printed electrodes can respond to artifacts like air enclosures in the wound cover or changes in the surrounding tissue. The described approaches are addressing one or two of the mentioned parameters. Most important issues to be solved are

- miniaturization and integratability,
- cost efficiency and
- high specificity by multiparametric ascertainment.

The presented solutions show high potential but also have to overcome these difficulties for practical implementation. These issues are one scope of our work.

In our research we focus on the ascertainment of one parameter which is strongly related to the infection status: the formation of neutrophil extracellular traps (NETs). NETs were first described by Brinkman *et al.* [14] and are caused by neutrophil granulocytes. Neutrophils are a kind of leukocytes and are the first cells arriving at the wound site after pathogen contact. Normally, they circulate within the blood in standby state. The immune reaction is triggered by different signal molecules, e.g. lipids of destroyed cells, growth factors or histamine, and results in migration of the neutrophils towards the wound. Once the neutrophils arrive, bacterial peptides initiate the NET-formation. It starts with the decomposition of the core membrane of the neutrophils resulting in a spreading of the chromatin and merging with the granules. Some minutes to hours after activation the cell membrane also dissolves and releases the bactericide NETs. They form agglomerates of fiber-like structures which are able to trap and immobilize microbes. This mechanism is an inherent immune response triggered by nearly every kind of pathogen. It occurs cumulative on wound sites and leads to characteristic changes in the wound exudate during infection. These properties mark NETs as a suitable parameter for infection monitoring in wounds.

2. Sensor concept

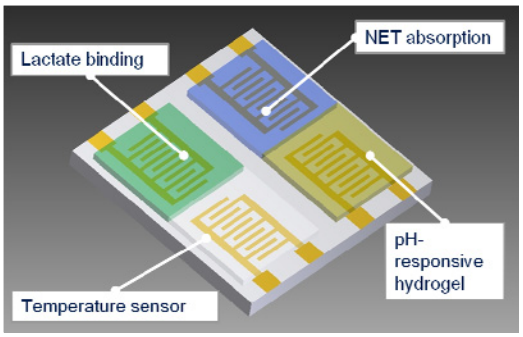

Fig. 1. Schematic of a multi-array impedance sensor with parameter selective coatings

One possible way to miniaturize the sensor system and to enhance cost efficiency is to use microfabrication standard processes. Our concept includes a sensor platform assembled as an electrode array with different functional coatings. This set-up is easy to fabricate with spin-coating, lithography and physical vapor deposition processes. Interdigitated electrodes have an advantageous field distribution with high field strengths at the surface of the sensor. Artifacts which can be a result of a large distributed field are avoided with this electrode configuration. Because of the direct application to the body, the excitation power of the impedimetric measurement should be low enough to avoid disturbances of biological functions but high enough to get a satisfactory measurement signal. Excitation frequencies that deliver a high signal output and are significant for the measurement task will be determined. Energy efficiency is enhanced by reducing the number of measurement points at certain frequencies. That makes the sensor more suitable for a full integration. On top of these electrodes several functional materials can be deposited like exudate absorbing layers, selectively structure-changing materials and materials to be decomposed by the presence of a measurement agent. A multiparametric ascertainment improves the

specificity to wound infections. The set-up and fabrication techniques permit the usage of biocompatible materials, such as ceramics and glasses for the substrate and gold for the electrodes. The functional coatings are polymers in the form of hydrogels or absorbent foams. For the detection of NET-formation a non-selective exudate-absorbing layer is required. The impedimetric NET-signal can be isolated by measuring at specific frequencies.

3. Measurement set-up

For the characterization of NET-formation we used impedance spectroscopy. The measurement principle is presented in Figure 2. Human neutrophil granulocytes were isolated from fresh blood samples and cultured on commercial interdigitated electrodes (Figure 2a). A poly-L-lysine layer assured immobilization of the neutrophils. After settling the cells in the measurement wells they were incubated at 37°C for at least 4 hours. The cell number was adjusted at 2 million cells per well. The electrode array was attached to an adapter which connects it electrically with an impedance analyzer (ScioSpec ISX-3). The measurements took place outside the incubator at room temperature. Former investigation proved that these conditions do not hinder NET-formation which was initiated by a chemical stimulant phorbol 12-myristate 13-acetate (PMA). To stimulate the granulocytes, the culture medium was replaced by medium containing 20 nM PMA. For the stimulation with S*almonella typhimurium*, the bacteria were added to the medium instead of PMA. The medium of negative control cultures was replaced with fresh medium to exclude influences of the medium exchange. It caused a shift of the impedance spectra in the same direction for both groups. The medium mixed with PMA has nearly the same impedance characteristic like the normal medium (0.3 % deviation) and did not influence the measurement accuracy. The impedance spectra for both stimulated and unstimulated cultures were recorded for 7 hours after medium exchange in certain time intervals. Figure 2 also illustrates the general principle of the impedance measurement of NET-formation. After stimulating the cells, a thick biofilm is formed (Figure 2b), which changes the complex impedance between the electrodes (Figure 2c, d).

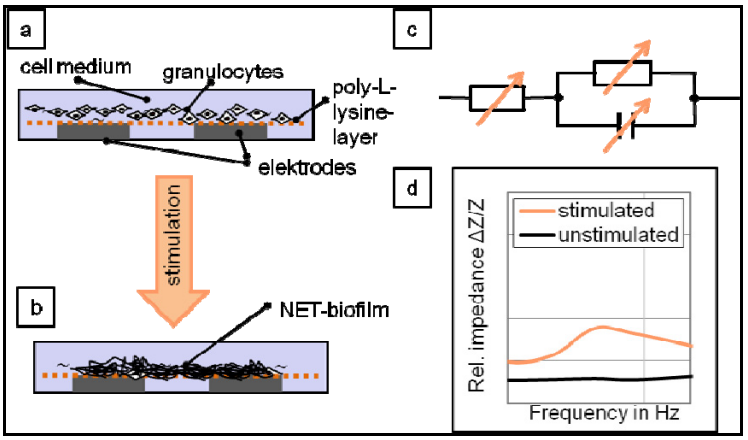

Fig. 2. Schematic of measurement procedure for impedance measurement of NET-formation, a) unstimulated cells on interdigitated electrodes, b) formation of a NET-biofilm on the electrodes after stimulation, c) simplified equilvalent circuit with changes after stimulation, d) impedance changes after stimulation

4. Results and discussion

4.1. Time course

Figure 3 shows a three-dimensional plot of normalized impedance and absolute phase angle change over frequency and time of stimulated and unstimulated cultures respectively. The first spectrum taken directly after medium exchange is used as reference. Real part and imaginary part of the complex impedance $\underline{Z}_n$ were measured and are given as follows:

$$\underline{Z}_n = \mathrm{Re}(\underline{Z}_n)+\mathrm{j}\cdot\mathrm{Im}(\underline{Z}_n) = Z_n\cdot\exp(-\mathrm{j}\cdot 2\pi\cdot f_n\cdot\varphi_n). \quad (1)$$

The relative change $\Delta Z_n/Z_0$ of the absolute impedance and the phase angle change $\Delta\varphi_n$ are calculated by

$$\Delta Z_n/Z_0 = (Z_n - Z_0)/(Z_0) \text{ and } \Delta\varphi_n = \varphi_n - \varphi_0 \quad (2)$$

with the excitation frequency f_n at n measuring points and Z_0 as the reference impedance. The absolute impedance rises up to 55 % after stimulating the culture. This maximum is reached after 1 to 4 hours. Maximum impedance occurs typically at a frequency of 17.5 kHz. Compared to the unstimulated control sample the impedance rise of stimulated samples is significant. The absolute impedance of the control sample changes with less than 4.8 %. Also in the phase deviation a substantial difference between stimulated and unstimulated neutrophils exists. As opposed to the impedance change which follows just one direction, the phase falls and rises at certain frequencies. Between the maximum and minimum of the phase curve the phase remains almost constant typically at about 18 kHz in average (see Table 2).

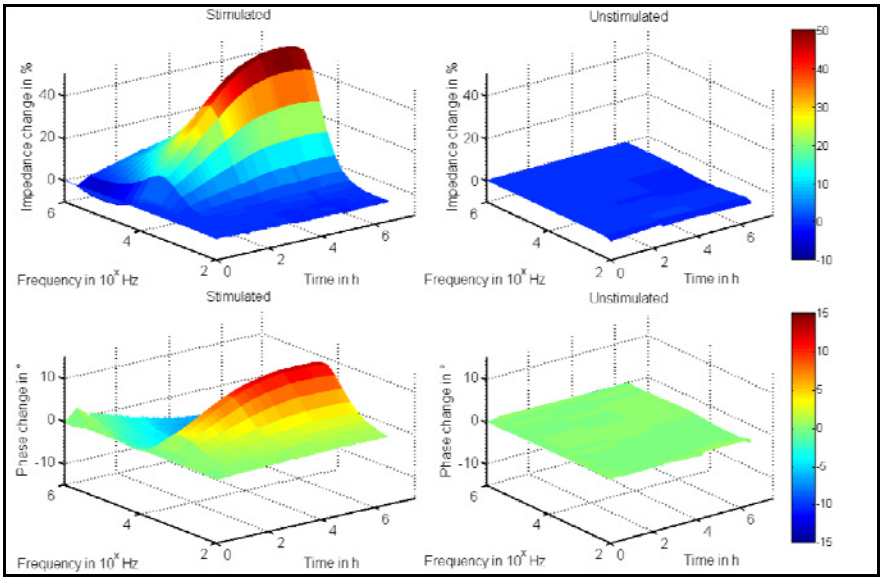

Fig. 3. Time course of impedance and phase change for stimulated and unstimulated neutrophils

Table 2. Characteristic values for impedance and phase changes

Parameter	Impedance change	Phase change	
	at maximum	at maximum	at minimum
Time to reach	(1...4) h	(0.5...5) h	(0.5...7.4) h
Value range	(28...55) %	(6.0...10.8)°	-(7.6…3.4)°
Average frequency	17.5 kHz	5.0 kHz	252.2 kHz
Deviation of control sample	(0.3...4.8) %	(0.5...2.3)°	-(2.1…0.4)°

The impedance change is nearly six times higher for stimulated samples compared to the unstimulated control sample. Hence, recording impedance at a frequency of 17.5 kHz is a proper parameter to detect NET-formation. For the phase change, at least two frequencies have to be record. The changes at minimum and maximum are at least 2.5 times higher than the control sample deviation. Determining a phase rise at 5 kHz and a fall at 252.2 Hz gives a strong hint for NET-formation. At certain frequencies the absolute impedance and phase do not differ much. These measurement points are suitable for stability measurements, for example in the higher (1 MHz) and lower frequency range (200 Hz). For other electrode configurations these values are different, but with the same remarkable trends.

4.2. Influence of cell number

Different cell numbers per well can influence impedance spectra and impedance changes. In natural environment the neutrophils flood the wound and excessively release NETs to entrap the pathogens. To check the reliability of the model environment created by *in vitro* experiments, effects of the cell number

as total number of cells per well (200 µL) were studied. For this experiment two wells per cell number were prepared. Figure 4 shows the time-dependent impedance change at 17.5 kHz.

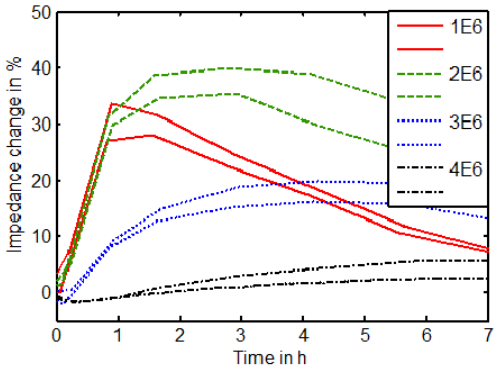

Fig. 4. Time course for stimulated neutrophils. Parameter: cell numbers per well

The standard cell number we used was amounted to 2 million cells per well. At the beginning the impedance increases strongly and reaches a maximum after 2 hours. Afterwards, the impedance decreases which is due to a natural decomposition of the NETs. For a lower cell number the reaction starts in the same way with a high rise of the absolute impedance. After nearly one hour the reaction stops and the impedance falls steeply. The lower maximum at the lower cell number occurs as expected and is due to less available cells for NET-formation. Chemical and biological agents attack the NETs more effectively because of a lower biofilm density. This leads to a faster degradation process and furthermore to a faster decrease of impedance after reaching its maximum. With higher cell numbers the reaction is slower and the maximum is reached later. This result reveals the limits of the experimental set-up. Due to a high density and a thick cell layer the medium diffuses slowly to the lower cell layers. The NET-formation at the sensor surface is delayed. At high cell density the NET-formation leads to a lower impedance increase because of a lower NET content per medium fraction. Comparing these results with the biological process as it takes place in a wound, the main difference is the constant supply of fresh cells. That keeps the NET-amount constant as the infection is in progress. A second difference is the direct application of neutrophils on the electrodes. In a wound environment the sensor has no contact with the cells but with the stimulated exudate already containing NETs. The ideal measurement conditions would be to measure just the NETs. Hence, 2 million cells per well are sufficient.

4.3. Stimulation with Salmonella typhimurium

Real infection with pathogens was simulated by stimulating the cells with PMA. To check if the neutrophils react in the same way with bacterial stimulation we used *S. typhimurium* with a multiplicity of infection of 25 as infection agent. Figure 5 compares stimulation with *S. typhimurium* and with PMA. Similarities in impedance changes can be found in the frequency of the maximum, which is at 17.6 kHz for *S. typhimurium* (PMA: 17.5 kHz). The intensity is also similar or even higher with 39 % on average (PMA: 34.6 %). The major difference can be found in the dynamics. The impedance reaches its maximum

much faster after 1.3 h (PMA: 3.4 h). The reason for this deviation depends on various factors, e.g. a faster response of neutrophils to bacteria than to the chemical stimulant. Because of a similar behavior concerning intensity of impedance change and characteristics, the model environment meets the requirements for further characterizations of the biological mechanisms. It should be kept in mind that reaction dynamics differ slightly.

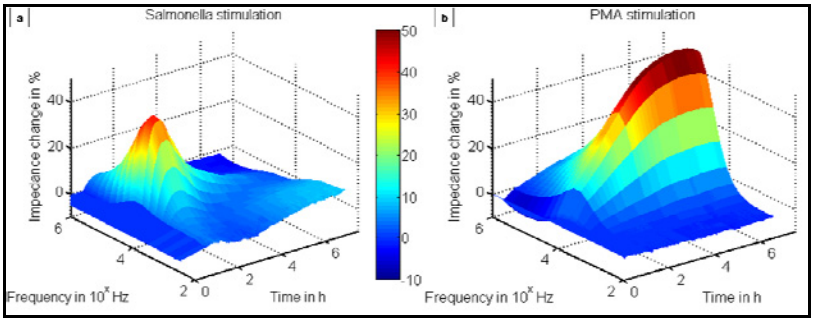

Fig. 5. Impedance changes for neutrophils stimulated a) with *S. typhimurium* and b) with PMA

5. Conclusions

Identification of wound infection indicators is challenging and not well investigated with respect to create reliable infection sensors. Therefore, we studied a multiparametric sensor based on impedimetric measurement. With this set-up we investigated the formation of neutrophil extracellular traps stimulated with bacteria or a chemical stimulant with different amounts of neutrophils. The spectra of cell cultures stimulated with PMA resulted in significant changes in absolute impedance and phase angle. These signals can be used for infection detection. The optimal cell number was investigated to match natural conditions and is at about 2 million cells. Comparing stimulation with PMA and *S. typhimurium* we found nearly the same behavior with similar reaction kinetics. Future investigations have to consider more detailed correlations between electrical measurements and biological process. This needs parallel microscopic observation to find the relationship between the several NET-formation steps and their structural characteristics measured with impedance.

Acknowledgements

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